Cobalamin-Dependent Radical S-Adenosylmethionine Enzymes in Natural Product Biosynthesis

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HIGHLIGHT

Cobalamin-Dependent Radical *S*-Adenosyl-L-Methionine Enzymes in Natural Product Biosynthesis

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This highlight summarizes the investigation of cobalamin (Cbl)- and radical S-adenosyl-L-methionine (SAM)-dependent enzymes found in natural product biosynthesis to date and suggests some possibilities for the future. Though some mechanistic aspects are apparently shared, the overall diversity of this family's functions and abilities is significant and may be tailored to the specific substrate and/or reaction being catalyzed. A little over a year ago, the first crystal structure of a Cbl- and radical SAM-dependent enzyme was solved, providing the first insight into what may be the shared scaffolding of these enzymes.

1. Introduction

In 1995, Kuzuyama and coworkers hypothesized the existence of an evolutionary relationship between the methyltransferases found in the biosyntheses of bialaphos (BcpD), fosfomycin (Fom3), and fortimicin KL1 (Fms7) (**Figure 1**) based on sequence similarity and the observation that all three enzymes apparently depended upon methylcobalamin (MeCbl) for activity.[1] In accordance with their apparent physiological functions, BcpD, Fom3, and Fms7 were discovered to contain protein sequences resembling the cobalamin-binding domain (CBD) of the enzyme cobalamin (Cbl)-dependent methionine synthase, MetH.

Fig. 1 Compounds whose biosynthetic pathways were initially postulated to include Cbl-dependent methylation by Kuzuyama, *et al*.[1] Key methyls are in red font.

MetH is a methylating enzyme that transfers a methyl group from 5-methyltetrahydrofolate to homocysteine, forming tetrahydrofolate and methionine.[2] MetH uses Cbl as an intermediary cofactor for transfer of the methyl group in an S_N2 nucleophilic substitution fashion, switching between oxidized +3 (MeCbl) and supernucleophilic +1 (Cbl(I)) states.[2] Occasionally, MetH becomes inactivated due to the loss of an electron from the supernucleophile, leading to formation of a +2 (Cbl(II)) state incapable of catalysis. When this occurs, addition of an electron restores the cofactor to the supernucleophilic state, and remethylation of Cbl(I) occurs using a molecule of SAM as the methyl group donor.[3-5] This reactivates MetH for further catalysis. Elegant structural studies have depicted snapshots of the domains of MetH in action.[5-10] However, structural characterization of the full MetH enzyme has remained elusive, so large conformational changes posited during catalysis and reactivation remain hypothetical.

A few years after Kuzuyama *et al.* had hypothesized the relationship between BcpD, Fom3, and Fms7, a major study by Sofia *et al.* in 2001 became the first to formally name the radical *S*-adenosyl-L-methionine (SAM) superfamily based upon the sequence similarity of 54 otherwise highly divergent protein sequences.[11] Sofia's work confirmed Kuzuyama's 1995 evolutionary hypothesis using a bioinformatics perspective, including all three of these enzymes and demonstrating genetic links between a variety of biosynthetic pathways together with a few catabolic pathways.[11]

Sofia *et al.*'s work identified two primary hallmarks of radical *S*-adenosyl-L-methionine (SAM) enzymes, though some exceptions have been found in the nearly two decades that have passed since this seminal paper.[11,12] First, all radical SAM enzymes contain a four-iron, four-sulfur ([4Fe-4S]) cluster, typically but not invariably bound by a canonical CxxxCxxC cysteine motif (**Scheme 1**). This cluster is required in a reduced +1 form to achieve the second shared characteristic, which is the generation of the 5'-deoxyadenosyl (5'-dAdo·) radical through homolytic cleavage of SAM upon donation of an electron from the reduced cluster (**Scheme 1**). Radical SAM enzymes use the 5'-dAdo· radical to abstract a hydrogen atom from the substrate. Thereafter, subsequent chemistry diverges widely among superfamily members. One notable exception to traditional radical SAM chemistry is embodied by the Cbl-dependent enzyme tryptophan methylase TsrM, which will be described in more detail below.

Scheme 1 Canonical radical SAM chemistry. Reversible homolytic cleavage of SAM mediated by the +1 form of the [4Fe-4S] cluster leads to the formation of methionine, the 5'-dAdo· radical, and the +2 form of the cluster.

As of 2014, ~114,000 radical SAM enzymes belonging to the PFAM PF04055 superfamily had been identified.[13] At that time, the Cbl-dependent group of enzymes was thought to be the largest subset within the radical SAM superfamily. A quick analysis of data in November, 2017 suggested that the number of total radical SAM sequences had grown to nearly 255,000, and it has been estimated

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that 7000+ of these radical SAM proteins contain CBDs.[14,15] The speed with which new genomes continue to be sequenced suggest that the number of these enzymes and potentially new functions will keep growing faster than laboratories are able to investigate them. Below is an attempt to summarize the results of studies in just the past 8 years (2011-2018) that illustrate the diversity of chemical reactions and mechanisms found in natural product biosynthetic pathways that are enabled by coupling radical SAM and Cbl chemistry.

2. *P*-methyl transfer

PhpK in phosphinothricin biosynthesis

In 2011, the first member of the Cbl-dependent radical SAM superfamily successfully purified and characterized was the *P*-methyltransferase from the soil bacterium *Kitasatospora phosalacinea*, which shares nearly complete sequence identity with a homologue, PhpK, from *Streptomyces viridochromogenes* and high identity with BcpD from *Streptomyces hygroscopicus*, the first *P*-methyltransferase to be identified.[16-18] *P*-methylation is required for the biosynthesis of L-phosphinothricin (PT) (**Figure 1**).[19,20] L-PT has antibiotic and herbicidal activity due to its inhibition of bacterial and plant glutamine synthetases.[21,22] It is typically found as the N-terminal residue of a secreted tripeptide. *Streptomyces* add two L-alanine residues, forming the natural product bialaphos, while *K. phosalacinea* adds the L-isomers of L-alanine and L-leucine to form phosalacine (**Figure 1**).[21-24]

Werner and Allen, *et al.*, were unable to isolate the *K. phosalacinea* enzyme in soluble form when expressed recombinantly in *Escherichia coli*.[16] The insolubility of this and related enzymes likely played a major role in the lack of publications in this area of enzymology. However, by modifying an existing literature procedure, Werner and Allen, *et al.*, successfully solubilized and refolded the enzyme.[25] Since PhpK was originally insoluble, purification resulted in apoenzyme, and loading of iron, sulphide, and MeCbl was required. EPR spectroscopy was used to show that the refolded enzyme indeed appeared to contain a [4Fe-4S] cluster that could be chemically reduced with sodium dithionite to the EPR-active +1 state. Using 2-dimensional (2-D) nuclear magnetic resonance (NMR) spectroscopy PhpK was shown to catalyse methyl transfer to an N-acetylated phosphinate substrate, 2-N-acetyl, 4-hydroxyphosphinobutanoate (or NAcDMPT) (**Scheme 2**).[16] In phosphinothricin producers including *S. viridochromogenes*, *S. hygroscopicus*, and *S. wedmorensis*, the N-acetyl modification serves as a resistance determinant.[26,27] Based on previous published studies, it is believed that PhpK can also accept N-acetylated tripeptide substrates,[20,28] but that has not yet been definitively demonstrated with purified enzyme.

Scheme 2 P-methylation reactions; added methyl group is in red font.

The work of Werner and Allen, *et al.*, suggested that, as predicted, the reaction was likely to proceed in a radical SAM-mediated fashion. Turnover was only observed in the presence of dithionite, suggesting that a reducing agent was necessary for the reaction.[16] Additionally, in accordance with previous studies, MeCbl was also required for turnover.[16,20] When labelled $^{13}CH_3$-MeCbl was supplied to the enzyme, 2-D NMR demonstrated that the direct source of the methyl group was MeCbl.[16,29] Under the conditions tested, PhpK was essentially suicidal and only capable of a single turnover.[16]

Over the past few years, the Booker laboratory has been at the forefront of Cbl-dependent radical SAM enzymology and led the charge towards determining the mechanisms of several enzymes in this class. More details about their work will be described herein. Very recently, this laboratory published the successful purification and production of small amounts (~1 mg/L culture) of soluble, recombinant PhpK using a combination of techniques, most notably the overexpression of Cbl uptake proteins.[30] In addition, enzyme produced in this manner is pre-loaded with the Cbl cofactor, primarily in the MeCbl state. However, PhpK overexpressed and purified via this procedure was inactive towards all substrates tested, leading Booker *et al.* to speculate that perhaps the physiological substrate for PhpK remains to be determined.[30] Clearly, more studies on *P*-methyl transfer by Booker, Wang, and/or others must be done to further unravel the mechanism behind this highly unusual biochemical reaction.

"SDen_1168" (now YgiQ) from *Shewanella denitrificans*

In 2008, during the Wang laboratory's early work in this area of enzymology, the open reading frame "sden_1168" (recently renamed ygiQ) from the marine denitrifying bacterium, *Shewanella denitrificans* OS217, as a potentially intriguing member of the Cbl-dependent radical SAM family. At the time, far fewer sequences had been published, and this particular sequence stood out for its relatively high shared similarity and identity with the *P*-methylases BcpD and PhpK and the Fom3 *C*-methylase involved in fosfomycin biosynthesis (*vide infra*). YgiQ is more similar to Fom3 than it is to BcpD/PhpK. The similarity shared between BcpD, PhpK, Fom3, and YgiQ suggested that YgiQ might function as either a *P*- or a *C*-methylase with an undetermined biological role, as *S. denitrificans* was not known to produce any small molecule antibiotics such as phosphinothricin or fosfomycin. At the time, the genome neighbourhood around this open reading frame also did not provide many clues as to the function of this protein.

During testing, no *C*-methylase activity from YgiQ was detected. Instead, Allen and Wang were surprised to find that the protein catalysed *P*-methyl transfer upon the BcpD/PhpK substrate, NAcDMPT (**Scheme 2**). Further, unlike PhpK (and presumably BcpD), YgiQ appears to accept the non-acetylated precursor to phosphinothricin, 2-amino, 4-hydroxyphosphinylbutanoate) as a substrate (**Scheme 2**).[31] As a result, the *Shewanella* enzyme is apparently able to produce phosphinothricin directly. This does not seem to be a favourable ability for the enzyme, considering that phosphinothricin's mode of action allows it to inhibit bacterial growth. Thus, Allen and Wang suspect that YgiQ fulfils some other biological function for this organism, perhaps catalysing a new and/or different *C*- or *P*-methylase reaction.[31] Indeed, work by the Metcalf group in 2013 suggests the *S. denitrificans* operon that contains YgiQ may be used to form a phosphonate or phosphinate natural product or cellular component.[31,32] More research is clearly needed in this area to determine the function of YgiQ and other related enzymes.

In studying YgiQ, Allen and Wang also published the first use of titanium (Ti) (III) citrate as a reductant for a Cbl-dependent radical SAM enzyme.[31] Ti citrate, with a reduction potential of ~-500 mV, had previously been used successfully as a one-electron reductant for other iron-sulfur enzymes.[33-35] The reduction potential of the

cluster in Cbl-dependent radical SAM enzymes is likely to be between -450 and -550 mV.[36-39] As described earlier, Werner and Allen, *et al.*, had success using sodium dithionite as a reducing agent with PhpK.[16] Around the same time, it was shown that dithionite could cause unwanted side reactions with cobalamin, generating a stable six-coordinate sulfoxide radical anion complex with Cbl in the +2 state that would likely inactivate a MeCbl-dependent enzyme.[40] Indeed, no YgiQ *P*-methylase activity was observed when excess dithionite was present in the assay, likely due to side reactions with MeCbl.[16] Since then, others have found that Ti citrate can be an appropriate *in vitro* reductant for related family members.[39]

3. *C*-methyl transfer

Fom3 in fosfomycin biosynthesis

As described earlier, the *C*-methylase Fom3 from fosfomycin biosynthesis was one of the first postulated members of the Cbl-dependent radical SAM family.[1] Fosfomycin (**Figure 1**) inhibits bacterial cell wall biosynthesis and is naturally produced by some *Streptomyces* and *Pseudomonas* species. Studies by several groups, resulting in the revision of multiple hypothetical pathways,[42-48] have recently and finally shown that cytidylyl-hydroxyethylphosphonate (CMP-HEP) is the physiological substrate for methylation, which results in the production of cytidylyl-hydroxypropylphosphonate (CMP-HPP) as product.[48]

Like PhpK, Fom3 from *Streptomyces wedmorensis* was initially purified from inclusion bodies and solubilized to provide high yields of enzyme with apparently low activity. Iron-sulfide analysis of hexahistidine-tagged protein suggested that the tag bound excess iron and sulphide compared with the expected 4:4:1 Fe:S:protein stoichiometry. EPR spectroscopy together with site-directed mutagenesis suggested the presence of the requisite reducible [4Fe-4S] cluster and thus implied the use of radical SAM chemistry for methylation. In contrast to PhpK, Allen and Wang used 1-dimensional ^{31}P to show weak methylase activity of hexahistidine-tagged Fom3 upon 2-hydroxyethylphosphonate (2-HEP) to produce 2-hydroxypropylphosphonate (2-HPP).[47]

In 2017, Sato and coworkers published a study of Fom3 in which their purified recombinant protein required no refolding and successfully methylated CMP-HEP to form CMP-HPP.[48] Using high performance liquid chromatography (HPLC), they showed evidence for the formation of HPP-CMP as well as production of 5'-dAdo in the presence of a chemical reducing system consisting of methyl viologen (MV) and reduced nicotinamide adenine dinucleotide (NADH) together with added SAM, MeCbl, and DTT, thus indicating radical SAM chemistry is at work. As observed for other Cbl-dependent radical SAM methylases (*vide infra*), SAH was also detected. This result provided evidence that MeCbl is consumed and regenerated via attack of the supernucleophile cob(I)alamin upon SAM to achieve multiple turnovers. In addition, Sato *et al.* suggested that perhaps remethylation of the Cbl cofactor occurs upon release of Cbl away from the enzyme.[48] Perhaps most strikingly, Sato *et al.* initially showed that Fom3 produces both (*R*)- and (*S*)- enantiomers of the methylated product in an approximately 1:1 ratio, leading them to conclude that Fom3 uses a secondary carbon radical formed on the HEP moiety of CMP-HEP to abstract the methyl group from MeCbl in a non-stereospecific fashion.[48] The apparent lack of stereoselectivity by Fom3 was a very unusual finding considering that one of the hallmarks of enzyme catalysis is stereospecificity due in part to their inherent chirality as a result of their L-amino acid compositions and configurations.

Model studies by Mosimann and Kräutler in 2000 were the first to demonstrate a chemical precedent for methylcorrinoids as methylating agents.[49] Based upon their own as well as others' results with model compounds,[50-52] Mosimann and Kräutler argued that radical methylation of an organic compound at a saturated carbon center should proceed stereospecifically with inversion of configuration. Since a second methyl transfer is required to regenerate MeCbl, configuration with respect to methionine as the "original" methyl group donor is believed to occur with net retention.[49] Thus, the results obtained by Sato *et al.* in 2017 do not mesh with chemical literature precedents.

In the past few months, three papers, including one from most of the authors of the 2017 Sato *et al.* publication, have shown that, in fact, Fom3 does catalyse a stereospecific methylation reaction that occurs with inversion of configuration.[53-55] The first of these papers, a 2018 study by Schweifer and Hammerschmidt, reaffirmed that when methionine is used as the primary methyl group donor in *Streptomyces fradiae in vivo*, methyl group stereochemistry is retained in the final fosfomycin antibiotic.[53] Hammerschmidt had previously shown over two decades ago that the pro-(*R*) hydrogen of 2-HEP is stereospecifically removed during fosfomycin biosynthesis.[56] The second and third papers by McLaughlin and van der Donk[54] and Sato *et al.*,[55] respectively, published essentially back-to-back this month, each independently came to the shared conclusion that methylation by Fom3 occurs with inversion of configuration and that only the (*S*)-isomer is formed (**Scheme 3**). These very recent results are consistent with the aforementioned model studies establishing chemical precedents for radical methylation involving Cbl.[49-52] Importantly, using overexpression methods similar to those used by the Booker laboratory,[30] McLaughlin and van der Donk also showed that the methyl groups transferred by Fom3 originate from SAM and use MeCbl as an intermediary cofactor.[54]

Scheme 3 Reported stereochemical outcomes and abbreviated mechanistic proposals for Cbl-dependent radical SAM enzymes. Panel A: Fom3 reaction[54,55]; Panel B: GenK reaction with (top) methyl radical transfer or (bottom) ketyl radical anion formation[57]; Panel C: TsrM reaction with a polar mechanism[58]. Methyl groups added are in red font.

GenK in gentamicin biosynthesis

In 2013, characterization of the Cbl-dependent methyltransferase GenK by the Liu group was published.[59] Chronologically, this was the third *C*-methylase of the Cbl-dependent radical SAM family to be identified; the second, TsrM, will be described in much more detail below. Like Fom3, GenK was proposed to catalyse the *C*-methylation of a hydroxylated sp^3 methylene, converting the compound $GenX_2$ to G418 in the gentamicin biosynthetic pathway of *Micromonospora echinospora*.[60] Gentamicins are aminoglycosides with clinically applicable antibiotic activities (**Figure 2**). Kim *et al.* found that, like PhpK, YgiQ, and Fom3, GenK was insoluble and successfully refolded and reconstituted active enzyme.[59] They found that flavodoxin, flavodoxin reductase, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) or MV with NADPH were effective reducing systems, while they obtained no activity with ditihionite as a reducing agent. Importantly, their group was the

first to report detection of 1:1 5'-dAdo and SAH during the course of the reaction.[59] Thus, they provided the first evidence that a member of this family of enzymes uses radical SAM chemistry, resulting in the production of the 5'-dAdo· radical. In addition, their results indicated that GenK uses Cbl as a cofactor, transferring the methyl group through as-yet uncharacterized chemistry and using SAM in its "traditional" role of nucleophilic substitution methylation to regenerate the Cbl cofactor. Thus, GenK appears to require 2 molecules of SAM for turnover: one for radical SAM chemistry, one for methylation.[59] The stoichiometry of 2 moles of SAM to 1 mole each of 5'-dAdo and SAH is a critical recurring theme among the remainder of the Cbl-dependent radical SAM enzymes that have been studied.

Fig. 2 Other representative natural products singly C-methylated by Cbl-dependent radical SAM enzymes. Key methyls are in red font.

Recently, the Liu group showed that methylation of the $GenX_2$ substrate occurs via retention of configuration (**Scheme 3**, panel B).[57] GenK is stereoselective for the *pro*-(*R*) hydrogen atom at C6', and the final product has the (*R*)-configuration at C6'.[57] This observation somewhat surprisingly differs from both the Fom3 result, from studies of the related bottromycin methylase(s), and from the chemical literature precedent.[49-53,61] In all of these other cases, inversion of configuration results upon methylation. Feeding studies and enzymatic characterization of Fom3 have shown inversion of configuration in which the pro-(*R*) hydrogen of the substrate is removed and subsequent methylation yields the (*S*)-configured product.[53-55] Though the bottromycin methylase has yet to be purified and/or mechanistically characterized, similar feeding studies performed on the bottromycin producer, *Streptomyces bottropensis*, led to the hypothesis that the bottromycin methylase(s) operate(s) via inversion of configuration.[61] Evolution has apparently tailored different mechanistic strategies depending upon the needs of the organism.

Alternative substrates were used by the Liu group to further investigate catalysis by GenK.[57] Two potential mechanisms for the GenK reaction involving either transfer of a methyl radical or formation of a ketyl intermediate that could attack MeCbl using S_N2 chemistry remain under consideration (**Scheme 3**, panel B).[57,59] Transfer of a methyl radical in a constrained fashion in which the MeCbl sits next to rather than above or opposite the carbon center is currently the more chemically favoured mechanism. However, attack by a ketyl intermediate has not yet been completely ruled out. Though further mechanistic characterization of enzymes like GenK that ideally provides more evidence for a stable Cbl(II) intermediate and/or a stable substrate radical remains to be done, it seems increasingly likely that Cbl-dependent radical SAM methylases are capable of formal methyl radical transfer.

TsrM in thiostrepton biosynthesis: Methylation of an sp^2 carbon without radical chemistry?

TsrM is required for the biosynthesis of the natural product thiostrepton by *Streptomyces laurentii*.[62] Thiostrepton (**Figure 2**) is a thiopeptide antibiotic that falls into the general category of ribosomally synthesized and post-translationally modified peptides (RiPPs). Mechanistic studies of TsrM, the Cbl-dependent radical SAM methyltransferase responsible for methylation of L-tryptophan at the C2 position of the indole ring, have been published the most extensively of any of the Cbl-dependent radical SAM enzymes with five total papers beginning in 2012.[39,58,63-65] These papers differ to some extent in their results and in their interpretation of their and/or others' results. The consensus among these studies is that despite containing the canonical CxxxCxxC moiety found in radical SAM enzymes, TsrM does not display typical superfamily behaviour in that it apparently does not use the 5'-dAdo· radical as part of its catalytic mechanism.[39,58,63,64] Despite several attempts, 5'-dAdo has not been detected in any published TsrM studies, while SAH – a hallmark of nucleophilic substitution methylation reactions – has been isolated.[39,63,65] The stoichiometry of SAH compared with SAM appears to be 1:1, rather than the 1:2 observed for GenK.[39,63] This suggests that all of the SAM consumed is used for methylation of tryptophan. TsrM has also been shown to bind a [4Fe-4S] cluster using its radical SAM cysteine motif.[39,63] However, the enzyme is active in the absence of reducing agents that should theoretically be required to reduce this cluster to the +1 state required for enzymatic activity.[39,58,63,64] In fact, the addition of reducing agents apparently slows enzyme activity by approximately half instead of improving activity.[63] Site-directed mutagenesis showed the [4Fe-4S] cluster is required for methylase activity but is not required for the production of SAH.[63]

The mechanism for the unusual methylation catalysed by TsrM remains under investigation and debate. Very different reaction mechanisms are favoured by each group studying this enzyme.[58,64] The sum of the experimental evidence appears to favour a proposal by Booker's group of two polar S_N2 displacements (**Scheme 3**, panel C).[58] Booker's mechanistic hypothesis is consistent with a previous report indicating that the methyl group of SAM is transferred with retention of configuration with respect to L-methionine,[66] though obviously neither tryptophan nor 2-methyltryptophan are chiral at C2 due to aromaticity. First, N1 of the tryptophan ring, which has been shown to be absolutely required for catalysis, is deprotonated, activating C2 of Trp. The π electrons of the ring serve as a nucleophile, attacking MeCbl to form Cbl(I) and a methylated tetrahedral intermediate. Deprotonation of C2 and reprotonation of N1 results in the final methylated product and restores sp^2 character. Attack of Cbl(I) upon SAM remethylates the cofactor.[58]

The second possible mechanism proposes homolytic cleavage of MeCbl to form a methyl radical and Cbl(II).[64] Transfer of the methyl radical to tryptophan accompanied by concomitant loss of both a proton, presumably to solvent, and transfer of an electron, presumably to the [4Fe-4S] cluster, result in the final methylated product.[64] This reduced cluster could then be used to reduce Cbl(II) to Cbl(I), which could remethylate the cofactor back to the active MeCbl state by attacking SAM.[64] The observed production of SAH corresponds with the remethylation event.[64] Booker's group argues against homolytic cleavage of MeCbl based on the previous stereochemical results showing retention of configuration relative to L-methionine.[39,66] They state racemization of the product should be the expected result of methyl radical addition to an sp^2 carbon atom. It was also initially proposed by Pierre *et al.* that one role of the [4Fe-4S] cluster might be to bind tryptophan.[63,64] However, that possibility was eliminated when the Booker group showed spectroscopically that the addition of Trp did not change the local environment of the cluster.[39]

A significant breakthrough achieved by each group investigating TsrM was successful purification of the first soluble member of this enzyme family without refolding. Each laboratory employed a

different method to purify this protein. The Berteau group was able to obtain soluble TsrM as a strep-tagged fusion.[63] Booker's group could not duplicate this result and instead used a hexahistdine-tagged small ubiquitin-like modifier (SUMO) protein fusion.[39,65] In addition, the Booker laboratory expressed the protein together with genes used for iron-sulfur cluster assembly and in M9-ethanolamine minimal media supplemented with hydroxocobalamin (HOCbl).[67-69] Notably, not only was the preparation from the Booker laboratory soluble, their TsrM preparation yielded the first superfamily member to be reported nearly fully loaded with cobalamin at an approximate 1:1 stoichiometry.[39] As a result, they were the first to report assaying their enzyme preparation without adding some form of Cbl. The successful efforts of the Berteau and Booker groups in purifying TsrM without refolding have been modified to gain information about other Cbl-dependent radical SAM enzymes.[14,41,70]

Despite the intensive study of this enzyme by multiple groups over the past 6 years, the chemical rationale for the use of a radical SAM scaffold for non-radical SAM chemistry remains puzzling. Further work, such as the elucidation of the structure of TsrM and/or the study of other sp^2-methylases from the Cbl-dependent radical SAM family, such as CloN6 (*vide infra*), may provide additional insight as to how and why these enzymes require both Cbl and SAM for catalysis without employing the 5'-dAdo· radical.

4. Multiple methylations upon a single substrate

Recently, four Cbl-dependent radical SAM methylases have been shown to catalyse more than one methylation upon a single substrate. These studies further highlight and demonstrate the already amazing versatility of this members of this enzyme family. In addition, the ability to catalyse multiple, sometimes processive, methylations poses interesting enzymatic problems that remain unsolved. How do these four enzymes accommodate their substrates? What is the stereochemistry of the reactions catalysed, and how do these enzymes control the stereochemical fate of their products?

Fig. 3 Natural products in which multiple methylations of the same substrate are catalyzed by one enzyme . Key alkyl groups are in red font.

ThnK in thienamycin biosynthesis

In 2015, a collaborative effort led by Booker and Townsend led to the first illustration of consecutive methyltransferase activity by the enzyme ThnK during the production of the carbapenem antibiotic thienamycin (**Figure 3**) by *Streptomyces cattleya*.[70] This group successfully cloned and overexpressed ThnK with a hexahistidine-tag under the growth conditions successfully employed for the study of TsrM. Testing of a variety of substrates resulted in the finding that a (2*R*,3*R*,5*R*)-carbapenem precursor carrying a pantetheine side chain substitution gave the best turnover. Both an ethylated and a methylated product, in lesser quantity, were isolated, demonstrating that this enzyme catalyzes sequential methylation reactions. The results of studies using deuterated d_3-SAM confirmed this hypothesis. This work showed that the CD_3 label was transferred first to form the methylated product (M+3 *m/z*) and again to form the ethylated product (overall M+5 *m/z*).[70]

As described above for Fom3[48] and GenK,[59] both 5'-dAdo and SAH were isolated as products in an essentially 1:1 ratio, providing evidence for the use of SAM in both radical and nucleophilic substitution mechanisms during catalysis.[70] Site-directed mutagenesis replacing the cysteines of the canonical radical SAM CXXXCXXC motif with alanines abolished the ability of the enzyme to form 5'-dAdo, though a small amount of SAH could still be formed. For these studies, MV with NADPH functioned as the best reductants for the cluster relative to dithionite and flavodoxin.[70]

CysS in cystobactamid biosynthesis

The Cbl-dependent radical SAM enzyme CysS was recently described as being responsible for the formation of branched groups during the biosynthesis of the cystobactamids (**Figure 3**), a group of antibacterial compounds synthesized by several myxobacteria.[71,72] The hypothesis was that CysS was responsible for methylating a single methyl ether to give subsequent ethyl (1st methylation), isopropyl (2nd methylation), and *sec*-butyl (3rd methylation) ethers. An *in vivo* experiment showed incorporation of ^{13}C from labelled $^{13}CH_3$-methionine into cystobactamid 919-1, suggesting that all of the carbons of the isopropyl groups were likely derived from SAM. Next, Begley's group successfully cloned and overexpressed CysS from *Cystobacter* as His-tagged protein together with iron-sulfur biosynthetic enzymes in the absence of cobalamin, resulting in soluble CysS that did not require refolding. They tested a few substrates derived from the 3-methyoxy-4-aminobenzoic acid framework believed to serve as a building block for cystobactamid biosynthesis. They observed ethyl, isopropyl, and both *sec*- and *tert*-butylation of some substrates after incubation with the enzyme. Similar to ThnK,[70] Begley's laboratory found that pantetheinylated substrates led to the best CysS activity.[71] Though a flavodoxin reducing system was needed to improve activity, these researchers noted the presence of low levels of two reaction products in the absence of reductant. 5'-dAdo, SAH, and the ethyl and isopropyl ether products were detected in a 2:2:1.4:0.3 ratio, suggesting that radical SAM chemistry occurred and that each enzyme equivalent was capable of 2+ turnovers. Begley's group has proposed sequential abstraction of a substrate hydrogen atom followed by the transfer of a methyl radical as a potential mechanism for the CysS-catalysed series of methylations leading to these branched compounds.[71] Determining how and/or why CysS is capable of methylating substrates to varying extents and the active site architecture that allows these methylations to occur in a potentially processive manner are two directions that are particularly intriguing for further study.

PoyC and PoyB in polytheonamide biosynthesis

Like thiostrepton, the polytheonamides (**Figure 3**) are classified as RiPPs.[73] These complex, fairly large, 49-amino acid peptides are highly cytotoxic to some mammalian cell lines and are therefore of potential interest as anti-tumour agents.[74] These compounds are produced by *Candidatus* Entotheonella factor, a so-far unculturable bacterial symbiont of the marine sponge *Theonella swinhoei.*[73,75] Separate, nearly simultaneous studies by Parent, *et al.*[41] and Freeman, *et al.*[75] demonstrated the methylase function of PoyC in polytheonamide biosynthesis. The experimental results from these two groups differ to some extent, with Parent's work being primarily *in vitro* using purified enzyme and a modified and

truncated polytheonamide precursor[41] while Freeman's work capitalized upon expression in *Rhizobium leguminosarum* and the use of full length polytheonamide precursors.[75]

Both groups appear to agree upon the ability of PoyC to catalyse the methylation of the valine side chain, essentially yielding a t-butyl glycine residue as a result.[41,75] Parent *et al.* successfully cloned and overexpressed PoyC using a modification of Booker's TsrM methodology.[39,65] To improve the solubility of the proposed substrate, this group added an N-terminal pentalysine residue to the first 15 residues of the polytheonamide peptide precursor, yielding a more tractable 20-amino acid peptide.[41] After testing this peptide in the presence of Ti citrate (as the reductant), SAM, and the enzyme, they found that amino acid 19, V14 of the precursor, was the target of methylation and had to be in the L-configuration for catalysis to occur.[41] Transfer of a deuterated methyl group to the product from CD_3-SAM confirmed the source of the methyl group. The apparent activity of the enzyme was quite poor based upon the low conversion of product (~6%). As observed for other enzymes in this family, both 5'-dAdo and SAH were observed in an approximately 1:1 ratio, reinforcing the recurring mantra that one molecule of SAM is required for radical chemistry and another is required for methyl transfer.[41] One somewhat significant difference between PoyC and the other enzymes in this family that have been characterized is that it has a CX_7CXXC motif rather than the classic CXXXCXXC cysteine motif. Nevertheless, Parent *et al.* showed through spectroscopy and site-directed mutagenesis that PoyC uses this modified cysteine motif to bind a [4Fe-4S] cluster. Further, the cluster is apparently required by PoyC for production of both 5'-dAdo and SAH.[41]

The work done by Freeman*, et al.* in *R. leguminosarum* also showed that PoyC was capable of methylation L-valine residues.[75] However, unlike Parent, *et al.*,[41] they did not observe methylation at V14. Instead, they observed methylation of valine residues found at substrate residues 5-7, 9, 10, and 21.[75] All but the last would have been present in the substrate used by Parent *et al.*,[41] though they are clearly shifted C-terminally relative to the native substrate due to the presence of the added N-terminal pentalysine. Other residues, including I3 and T1, appeared to be methylation targets for PoyC in *R. leguminosarum*.[75] The T1 methylation required the co-expression of PoyF, another enzyme in the *Candidatus* Entotheonella biosynthetic pathway. Freeman *et al.*, reported that, in general, zero or one methylation was observed, though up to four methylations were occasionally detected. Overall, they concluded that PoyC was likely responsible for all of the methylations found in amino acids 1-21 of the polytheonamide precursor.[75]

In addition, the work of Freeman and colleagues showed that PoyB, another member of the Cbl-dependent radical SAM family, is also capable of C-methylation.[75] The primary role of PoyB appears to be methylation of Q23, though it is also linked to methylation of V31 and M45. The activity of PoyB in *R. leguminosarum* appeared to be relatively poor in comparison with PoyC. The authors thus concluded that PoyB was likely responsible for methylating residues found in the C-terminal half of the polytheonamide precursor.[75] Unfortunately, to date, PoyB has apparently been intractable for detailed study because it is not expressed in soluble form according to Parent, *et al.*[41] In total, the polytheonamides contain 17 putative C-methylations, 13 of which were successfully detected by Freeman, *et al.* in *R. leguminosarum*.[75] Future studies will hopefully reconcile the differences in activity observed between the two laboratories as well and in the final natural products, and structural investigation may provide insight as to how these proteins recognize and methylate their peptide substrates.

5. Non-methylases

OxsB in oxetanocin A biosynthesis

The first and, to date, only crystal structure of a Cbl-dependent radical SAM enzyme, OxsB, was published in 2017.[14] Intriguingly, this enzyme is not a methylase, thus also making it the first non-methylase in this family to be described and characterized. OxsB is one of four clustered genes found in *Bacillus megaterium* NK84-0128 that is required for the production of the antiviral compound oxetanocin A (OXT-A),[76] and it was identified early on by Sofia *et al.* as a member of the radical SAM superfamily.[2] Like other Cbl-dependent radical SAM enzymes, OxsB was known to encode both the canonical CXXXCXXC motif and a CBD that showed similarity to that found in methionine synthase. However, OXT-A does not contain any methyl groups, thus obviating the need for a methyltransferase in its biosynthetic pathway.

Through rigorous testing of a wide variety of substrates together with knockout work in *B. megaterium*, Bridwell-Rabb *et al.* discovered a variety of intriguing findings.[14] First, OxsB needed to be isolated. It was cloned and overexpressed as a soluble poly-histidine-tagged maltose binding protein (MBP) fusion protein; subsequent purification and protease treatment removed the extraneous fusion. OXT-A formation in an engineered *B. megaterium* strain was only observed when both OxsA and OxsB were expressed; no activity could be observed when OxsB was expressed alone either *in vitro* or *in vivo*.[14] OxsA functions as a phosphohydrolase leading to the final OXT-A product (**Scheme 4**).[14,77]

Scheme 4 OxsB-catalyzed ring contraction (di- and triphosphate substrates are also accepted) and OxsA-mediated production of OXT-A.

When potential substrates were tested with a combination of both proteins *in vitro*, enzymatic activity was observed using deoxyadenosine mono, di, and triphosphates (dAMP, dADP, and dATP) (**Scheme 4**).[14] Importantly, the form of Cbl used for assays was HOCbl instead of MeCbl, and there is no apparent need or role for the methylated form of this cofactor in this reaction. A single product, the ring-contracted aldehyde monophosphate (**Figure 4**), was observed regardless of the substrate.[14] As for several other enzyme family members, the combination of MV with NADPH was an effective reducing agent, which was required for activity as would be expected for radical SAM chemistry. 5'-dAdo was detected during the course of the reaction, providing further evidence for radical SAM chemistry.[14] A mechanism for ring contraction was proposed in which catalysis is initiated with 5'-dAdo· mediated hydrogen atom abstraction at C2'. The resulting substrate radical rearranges, and the subsequent loss of an electron and a proton result in the product aldehyde.[14] The role of Cbl in this reaction is currently unclear. Possibilities include coordination of a radical intermediate and/or serving as an electron donor and/or sink.[14] More work is needed to distinguish between these intriguing and potentially novel chemical rationales for the requirement of Cbl in this reaction mechanism.

In addition to delineating the activity of OxsB, Bridwell-Rabb and colleagues successfully obtained several structures of the

protein including the apoenzyme, enzyme containing the [4Fe-4S] cluster and aquocobalamin, and enzyme containing cluster, Cbl, and SAM (**Figure 4**).[14] The enzyme was not crystallized with bound substrate or bound product. Nevertheless, the resulting OxsB structures shed light on the functions of the widely divergent members of this enzyme family, incorporating facets found in both radical SAM and Cbl-dependent enzymes with some modifications. OxsB crystallizes as a single subunit comprised of four domains.[14] At present, the function of the N-terminal domain (**Figure 4**, domain I) is unknown, while the last domain, a C-terminal helix bundle (**Figure 4**, domain IV), may serve to close the active site for catalysis upon substrate-binding.[14]

Fig. 4 Structure of apo-OxsB, adapted from reference 14. N-terminal domain (I), yellow; CBD (II), magenta; TIM barrel/radical SAM domain (III), cyan; C-terminal domain (IV), royal blue; [4Fe-4S], gray; aquocobalamin, orange; SAM, lime green.

The second domain is the CBD, a Rossmann fold that shares significant structural similarity with the MetH CBD despite relatively low (14%) sequence identity[14] (**Figure 4**, domain II). OxsB does not use the classic DXHXXG "His-on" Cbl binding motif to bind Cbl. Instead, a flexible 14-residue loop moves and caps the Cbl-binding site when Cbl is present.[14] While the lack of classic "His-on" binding is different than MetH, it is not surprising. TsrM has been shown spectroscopically to bind Cbl in a dimethylbenzimidazole (DMB) base-off, His-off manner,[39] and sequence alignment of Cbl-dependent radical SAM family members does not indicate the presence of a conserved histidine residue to fulfil the "His-on" function.

The third domain is a partial triose phosphate isomerase (TIM) barrel that binds SAM for radical chemistry[14] (**Figure 4**, domain III).[14] Variations of this fold have been found in all solved radical SAM structures to date. In OxsB, this domain shows some interesting differences compared with other solved radical SAM structures that appear to allow it to perform Cbl-dependent chemistry. Some outward displacement of strands and helices expand the fold to accommodate Cbl. In addition, two conformations for SAM binding to the [4Fe-4S] cluster are observed. In one, SAM is oriented for homolytic cleavage and subsequent radical chemistry, but in the other, electron transfer seems unlikely. In the latter conformation, the methyl group is found close to (approximately 5.8 Angstroms away from) Cbl.[14] The authors note that the latter conformation is unlikely to be catalytically relevant to OxsB, but it provides a very nice parallel to the reactivation complex of MetH.[12,14] Though OxsB is not itself a methylase, this binding mode is consistent with and appears to provide clues as to how the methylases in this family conduct chemistry.

6. Conclusions and outlook: Other potential roles for cobalamin and radical SAM-dependent enzymes

An amazing amount of work has been done in the Cbl-dependent radical SAM field in this decade. Several other reviews have already been written that address this area of research, and the author encourages the reader to peruse earlier reviews for more mechanistic and/or enzymological details.[78-81] Indeed, it seems likely that in the timespan between the writing and publication of this review, at least one new enzyme in this huge family will be described in the literature. In fact, several publications about Fom3 and PhpK in the past 5 months have contributed significantly towards the mechanistic landscape and the future study of this enzyme class.[30,53-55] That said, a schematic depicting "typical" Cbl-dependent radical SAM methylation, which has been the most studied reaction class to date, is depicted below in **Scheme 5**. The majority of characterized enzymes in this family have been shown or are believed to require two molecules of SAM per round of catalysis. One is used to form the canonical 5'-dAdo· radical, while the other is used as the source of a methyl group. Thus, the products of catalysis include one molecule of 5'-deoxyadenosine and one molecule of SAH. TsrM and OxsB do not use this mechanism, and further evidence supporting this general mechanism and/or alternatives to it still needs to be obtained.

Scheme 5 General mechanistic proposal for Cbl-dependent radical SAM enzymes; TsrM and OxsB are exceptions.

Clearly this vast group of enzymes is capable of amazingly diverse chemistry to achieve challenging biochemical objectives. There is almost certainly insufficient funding and manpower to investigate the tens of thousands of Cbl-dependent radical SAM enzymes which remain unstudied. One major area for future research would be one of the many homologues of TsrM because of this enzyme's apparent lack of canonical radical SAM chemistry.[39,58,63,64] A few of the many other unaddressed possibilities that might shed light on results obtained to date and/or look at different classes of natural products are listed below for the reader's consideration.

Fig. 5 Examples of the diversity of natural products whose biosynthetic pathways contain putative Cbl-dependent radical SAM enzymes whose catalytic mechanisms have yet to be successfully investigated. Key methylation positions are in red font. The Cbl-dependent radical SAM reaction in bacteriochlorophyll biosynthesis is believed to be cyclization; the bond formed is marked bold red for easier visualization and does not reflect stereochemistry.

CloN6 is a putative Cbl-dependent radical SAM enzyme found in the biosynthesis of the aminocoumarin antibiotic clorobiocin (**Figure 5**) by *Streptomyces roseochromogenes*. This enzyme was first identified 16 years ago, making it a relatively "old" member of this superfamily.[82] However, it remains poorly investigated from a mechanistic perspective. Like TsrM, the substrate for CloN6 appears to be an sp^2-hybridized carbon atom. Successful characterization of this enzyme may shed some additional light on the TsrM reaction mechanism, specifically whether its apparent lack of usage of the 5'-dAdo· is common among members of this family that catalyse sp^2-hybridized *C*-methylation.

The fortimicin (**Figure 1**) methyltransferase Fms7 is another enzyme whose investigation may shed further light onto the mechanisms of sp^3-hybridized *C*-methylation.[1,11] Indeed, Fms7, like BcpD/PhpK and Fom3, could be considered one of the founding fathers of the Cbl-dependent radical SAM family. Work in the area of sp^3 carbon methylations, whether upon Fms7 or another related methylase, would likely assist in reconciling and/or explaining the apparent mechanistic differences observed thus far between the Fom3 and GenK enzymes. The structure of the demethylated

precursor is likely somewhat challenging to address from a synthetic perspective, but work on enzymes such as TsrM,[39] CysS,[71] and PoyC[41] suggest that truncated substrate analogues are often recognized by this class of enzymes.

The focus of published efforts in the field has clearly been upon natural products with antibiotic and other anti-infective activities. However, many other biosynthetic products are believed to contain methyl groups and/or other modifications that are mediated by Cbl-dependent radical SAM chemistry. In 2010, the *hpnP* gene was identified in *Rhodopseudomonas palustris* TIE-1 (and other bacteria) as potentially being responsible for C-2 methylation during the biosynthesis of hopanoids, multicyclic hydrocarbons that are believed to play a cholesterol-like role in stabilizing bacterial cell envelopes (**Figure 5**).[83,84] Though HpnP was identified quite some time ago, no further work with this enzyme has been published to date. As for Fms7, the proposed substrates are likely very challenging to work with and, since they are membrane associated and highly hydrophobic, may be largely insoluble.

Lastly, what about non-methylases in this enzyme family? This is a challenging area: as studies of OxsB have shown, unprecedented chemistry can result from Cbl-dependent radical SAM enzymes.[14] Unfortunately, however, in the absence of genomic context and/or other clues, it can often be difficult to hypothesize the activities of such enzymes. One non-methylase that was actually first identified by Kuzuyama *et al.* in 1995 and subsequently investigated by Gough *et al.* in 2000 that belongs to this family is BchE, the protoporphyrin IX cyclase (**Figure 5**).[1,11,85] This protein, which is required for anaerobic bacteriochlorophyll biosynthesis in some phototrophic bacteria, appears to require a Cbl cofactor and contains a radical SAM cysteine domain.[85-87] Thus, it appears likely – though in contrast to a previously proposed mechanism[85] – that BchE utilizes both compounds for a radical cyclization reaction.[87] The active site of BchE must be very intriguing indeed, as it must essentially accommodate a [4Fe-4S] cluster, SAM, and two porphyrins simultaneously. In fact, bacteriochlorophyll biosynthesis has also been shown to require two Cbl-dependent radical SAM enzymes for methylation, indicating that this anabolic pathway is replete with challenging, cofactor-laden chemistry.[88-90]

With continued persistence, sufficient resources, and the ever-improving variety of chemical tools available in the laboratory, the diverse field of Cbl-dependent radical SAM chemistry in natural product biosynthesis is surely one that can be pursued by both seasoned veterans and the next generations of scientists to come.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

1 T. Kuzuyama, T. Seki, T. Dairi, T. Hidaka, and H. Seto, *J. Antibiot.*, 1995, **48**, 1191.
2 R.V. Banerjee and R.G. Matthews, *FASEB J.*, **4**, 1450.
3 R.T. Taylor and H. Weissbach, *J. Biol.Chem.*, 1967, **242**, 1517.
4 K. Fujii, J.H. Gallivan, and F.M. Huennekens, *Arch. Biochem. Biophys.,* 1977, **178**, 662.
5 V.Bandarian, M.L. Ludwig, and R.G. Matthews, *Proc. Natl. Acad. Sci. USA,* 2003, **100**, 8156.
6 C.L. Drennan, S. Huang, J.T. Drummond, R.G. Matthews, and M.L. Ludwig, *Science*, 1994, **266**, 1669.
7 M.M. Dixon, S. Huang, R.G. Matthews, and M. Ludwig, *Structure*, 1996, **4**, 1263.
8 V. Bandarian, K.A. Pattridge, B.W. Lennon, D.P. Huddler, R.G. Matthews, and M.L. Ludwig, *Nat. Struct. Biol.*, 2002, **9**, 53.
9 J.C. Evans, D.P. Huddler, M.T. Hilgers, G. Romanchuk, R.G. Matthews, and M.L. Ludwig, *Proc. Natl. Acad. Sci. USA*, 2004, **101**, 3729.
10 M. Koutmos, S. Datta, K.A. Pattridge, J.L. Smith, and R.G. Matthews, *Proc. Natl. Acad. Sci. USA*, 2009, **106**, 18527.
11 H.J. Sofia, G. Chen, B.G. Hetzler, J.F. Reyes-Spindola, and N.E. Miller, *Nuc. Acids Res.*, 2001, **29**, 1097.
12 P.A. Frey, *Annu. Rev. Biochem.*, 2001, **70**, 121.
13 E. Akiva, S. Brown, D.E. Almonacid, A.E. Barber, 2nd, A.F. Custer, M.A. Hicks, C.C. Huang, F. Lauck, S.T. Mashiyama, E.C. Meng, D. Mischel, J.H. Morris, S. Ojha, A.M. Schnoes, D. Stryke, J.M. Yunes, T.E. Ferrin, G.L. Holliday, and P.C. Babbitt, *Nuc. Acids Res.,* 2014, **42**, D521.
14 J. Bridwell-Rabb, A. Zhong, H.G. Sun, C.L. Drennan, and H.-W. Liu, *Nature*, 2017, **544**, 322.
15 Enzyme Function Initiative – Enzyme Similarity Tool, http://efi.igb.illinois.edu/efi-est/
16 W.J. Werner, K.D. Allen, K. Hu, G.L. Helms, B.S. Chen, and S.C. Wang, *Biochemistry*, 2011, **50**, 8986.
17 D. Schwartz, N. Grammel, E. Heinzelmann, U. Keller, and W. Wohlleben, *Appl. Environ. Microbiol*., 2004, **70,** 7093.
18 T. Murakami, H. Anzai, S. Imai, A. Satoh, K. Nagaoka, and C.J. Thompson, *Mol. Gen. Genet.*, 1986, **205,** 42.
19 H. Seto, T. Sasaki, S. Imai, T. Tsuruoka, H. Ogawa, A. Satoh, S. Inouye, T. Niida, and N. Otake, *J. Antibiot.*, 1982, **36,** 96.
20 K. Kamigiri, T. Hidaka, S. Imai, T. Murakami, and H. Seto, J. Antibiot., 1992, **45,** 781.
21 G. Hoerlein, *Rev. Environ. Contam. and Tox*., 1994, **138**, 73.
22 C.J. Thompson and H. Seto, *Genetics and Biochemistry of Antibiotic Production*, 1995, 197.
23 S. Omura, M. Murata, H. Hanaki, K. Hinotozawa, R. Oiwa, and H. Tanaka, *J. Antibiot.*, 1984, **37**, 829.
24 S. Omura, K. Hinotozawa, N. Imamura, and M. Murata, *J. Antibiot*., 1984, **37**, 939.
25 W.P. Liu, I. Schiau, J.R. Cunningham, and S.W. Ragsdale, *J. Biol. Chem.*, 1993, **268**, 5605.
26 C.J. Thompson, N.R. Mowa, R. Tizard, R. Crameri, J.E. Davies, M. Lauwereys, and J. Botterman, *EMBO J.*, 1987, **6**, 2519.
27 W. Wohlleben, W. Arnold, I. Broer, D. Hilleman, E. Strauch, and A. Pühler, *Gene*, **70**, 25.

28 H. Seto, S. Imai, T. Tsuruoka, A. Satoh, M. Kojima, S. Inouye, T. Sasaki, and N. Otake, *J. Antibiot.*, 1982, **35**, 1719.
29 K. Hu, W.J. Werner, K.D. Allen, and S.C. Wang, *Magnetic Res. Chem.*, 2015, **53**, 267.
30 N.D. Lanz, A.J. Blaszcyk, E.L. McCarthy, B. Wang, R.X. Wang, B.S. Jones, and S.J. Booker, *Biochemistry*, 2018, **57**, 1475.
31 K.D. Allen and S.C. Wang, *BBA – Proteins and Proteomics*, 2014, **1844**, 2135.
32 X. Yu, J.R. Doroghazi, S.C. Janga, J.K. Zhang, B. Circello, B.M. Griffin, D.P. Labeda, and W.W. Metcalf, 2013. *Proc. Natl. Acad. Sci. USA*, **110**, 20759.
33 P. Lindahl, E. Munck, and S.W. Ragsdale, *J. Biol. Chem.*, 1990, **265**, 3873.
34 S. Menon and S.W. Ragsdale, *Biochemistry*, 1998, **37**, 5689.
35 G. Bender, E. Pierce, J.A. Hill, J.E. Darty, and S.W. Ragsdale, Metallomics, 2011, **3**, 797.
36 N.B. Ugulava, B.R. Gibney, and J.T. Jarrett, *Biochemistry*, 2001, **40**, 8343.
37 G.T. Hinckley and P.A. Frey, *Anal. Biochem.*, 2006, **349**, 103.
38 G.T. Hinckley and P.A. Frey, *Biochemistry*, 2006, **45**, 3219.
39 A.J. Blaszczyk, A. Silakov, B. Zhang, S.J. Maiocco, N.D. Lanz, W.L. Kelly, S.J. Elliott, C. Krebs, and S.J. Booker, *J. Am. Chem. Soc.*, 2016, **138**, 3416.
40 D.S. Salnikov, R.Silaghi-Dumitrescu, S.V. Makarov, R. van Eldik, and G.R. Boss, *Dalton Trans.*, 2011, **40**, 9831.
41 A. Parent, A. Guillot, A. Benjdia, G. Chartier, J. Leprince, and O. Berteau, *J. Am. Chem. Soc.*, 2016, **138**, 15515.
42 T. Hidaka, M.Goda, T. Kuzuyama, N.Takei, M.Hidaka, and H. Seto, *Mol. Gen. Genet.,* 1995, **249**, 274.
43 H. Seto and T. Kuzuyama, *Nat. Prod. Rep*., 1999, **16**, 589.
44 R.D. Woodyer, G. Li, H. Zhao, and W.A. van der Donk, *Chem. Comm. (Camb).*, 2007, **4**, 359.
45 L.J. Higgins, F. Yan, P. Liu, H.W. Liu, and C.L. Drennan, *Nature*, 2005, **437**, 838.
46 D. Yun, M. Dey, L.J. Higgins, F. Yan, H.W. Liu, and C.L. Drennan, *J. Am. Chem. Soc*., 2011, **133**, 11262.
47 K.D. Allen and S.C. Wang, *Arch. Biochem. Biophys.*, 2014, **543**, 67.
48 S. Sato, F. Kudo, S.-Y. Kim, T. Kuzuyama, and T. Eguchi, *Biochemistry*, 2017, **56**, 3519.
49 H. Mosimann and B. Kräutler, *Angew Chem. Intl. Ed.*, 2000, **39**, 393.
50 J.Z. Chrzastowski, C.J. Cooksey, M.D. Johnson, B.L. Lockman, and P.N. Steggles, *J. Am. Chem. Soc.*, 1975, **97**, 932.
51 D. Dodd, M.D. Johnson, and B.L. Lockman, *J. Am. Chem. Soc.*, 1977, **99**, 3644.
52 M.D. Johnson, *Acc. Chem. Res.*, 1983, **16**, 343.
53 A. Schweifer and F. Hammerschmidt, *Biochemistry*, 2018, **57**, 2069.
54 M.I. McLaughlin and W.A. van der Donk, *Biochemistry*, 2018, doi: 10.1021/acs.biochem.8b00616 [Epub ahead of print].
55 S. Sato, F. Kudo, T. Kuzuyama, F. Hammerschmidt, and T. Eguchi, *Biochemistry*, 2018, doi: 10.1021/acs.biochem.8b00614 [Epub ahead of print].
56 F. Hammerschmidt, *Liebigs Ann. der Chem.*, 1992, **6**, 553.
57 H.J. Kim, Y.-N. Liu, R.M. McCarty, and H.-W. Liu, *J. Am. Chem. Soc.*, 2017, **139**, 16084.
58 A.J. Blaszczyk, B. Wang, A. Silakov, J.V. Ho, and S.J. Booker, *J. Biol. Chem.*, 2017, **292**, 15456.
59 H.J. Kim, R.M. McCarty, Y. Ogasawara, Y.-N. Liu, S.O. Mansoorabadi, J. LeVieux, and H.-W. Liu, *J. Am. Chem. Soc.*, 2013, **135**, 8093.
60 J. Unwin, S. Standage, D. Alexander, T. Hosted, Jr., A.C. Horan, and E.M. Wellington, *J. Antibiot.*, 2004, **57**, 436.
61 J.L. Kellenberger, Ph.D. thesis, ETH, 1997.
62 W.L. Kelly, L. Pan, and C. Li, *J. Am. Chem. Soc.*, 2009, **131**, 4327.
63 S. Pierre, A. Guillot, A. Benjdia, C. Sandstrom, P. Langella, and O. Berteau, *Nat. Chem. Biol.*, 2012, **8**, 957.
64 A. Benjdia, S. Pierre, C. Gherasim, A. Guillot, M. Carmona, P. Amara, R. Banerjee, and O. Berteau, *Nat. Comm.*, 2015, **6**, 8377.
65 A.J. Blaszczyk, R.X. Wang, and S.J. Booker, *Methods Enzymol.*, 2017, **595**, 303.
66 P. Zhou, D. O'Hagan, U. Mocek, Z. Zeng, L.D. Yuen, T. Frenzel, C.J. Unkefer, J.M. Beale, and H.G. Floss, *J. Am. Chem. Soc.*, 1989, **111**, 7274.
67 R. M. Cicchillo, D.F. Iwig, A.D. Jones, N.M. Nesbitt, C. Baleanu-Gogonea, M.G. Souder, L. Tu, and S.J. Booker, *Biochemistry*, 2004, **43**, 6378.
68 H.D. Lanz, T.L. Grove, C.B. Gogoniea, K.H. Lee, C. Krebs, and S.J. Booker, *Methods Enzymol.*, 2012, **516**, 125.
69 V. Bandarian and R.G. Matthews, *Biochemistry*, 2001, **40**, 5056.
70 D.R. Marous, E.P. Lloyd, A.R. Buller, K.A. Moshos, T.L. Grove, A.J. Blaszczyk, S.J. Booker, and C.A. Townsend, *Proc. Natl. Acad. Sci. USA*, 2015, **112**, 10354.
71 Y. Wang, B. Schnell, S. Baumann, R. Muller, and T.P. Begley, *J. Am. Chem. Soc.*, 2017, **139**, 1742.
72 S. Baumann, J. Herrmann, R. Raju, H. Steinmetz, K.I. Mohr, S. Huttel, K. Harmrolfs, M. Stadler, and R. Müller, *Angew. Chem., Int. Ed.*, 2014, **53**, 14605.
73 M.F. Freeman, C. Gurgui, M.J. Helf, B.I. Morinaka, A.R. Uria, N.J. Oldham, H.G. Sahi, S. Matsunaga, and J. Piel, *Science*, 2012, **338**, 387.
74 T. Hamada, T. Sugawara, S. Matsunaga, and N. Fusetani, *Tetrahedron Lett.*, 1994, **35**, 719.
75 M.F. Freeman, M.J. Helf, A. Bhushan, B.I. Morinaka, and J. Piel, *Nat. Chem.*, 2017, **9**, 387.
76 N. Shimada, S. Hasegawa, T. Harada, T. Tomisawa, A. Fujii, and T. Takita, *J. Antibiot.*, 1986, **39**, 1623.
77 J. Bridwell-Rabb, G. Kang, A. Zhong, H.-W. Liu, and C.L. Drennan, *Proc. Natl. Acad. Sci. USA*, 2016, **113**, 13750.
78 Q. Zhang, W.A. van der Donk, and W. Liu, *Acc. Chem. Res.*, 2012, **45**, 555.
79 D.G. Fujimori, *Curr. Opin Chem. Biol.*, 2013, **17**, 597.
80 M.R. Bauerle, E.L. Schwalm, and S.J. Booker, *J. Biol. Chem.*, 2015, **290**, 3995.
81 S. Zhou, L.M. Alkhalaf, E.L.C. de los Santos, and G.L. Challis, *Curr. Opin. Chem. Biol.*, 2016, **35**, 73.
82 N. Mahanta, G.A. Hudson, and D.A. Mitchell, *Biochemistry*, 2017, **56**, 5229.L. Westrich, L. Heide, and S.M. Li, *ChemBioChem*, 2003, **4**, 768.
83 P.V. Welander, M.L. Coleman, A.L. Sessions, R.E. Summons, and D.K. Newman, *Proc. Natl. Acad. Sci. USA*, 2010, **107**, 8537.
84 H. Sahm, M. Rohmer, S. Bringer-Meyer, G.A. Sprenger, and R. Welle, *Adv. Microb. Physiol.*, 1993, **35**, 247.
85 S.P. Gough, B.O. Petersen, and J.O. Duus, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 6908.
86 S. Ouchane, A.S. Steunou, M. Picaud, and C. Astier, *J. Biol. Chem.*, 2004, **279**, 6385.
87 S.J. Booker, *Curr. Opin. Chem. Biol.*, 2009, **13**, 58.2007, **189**, 6176.
88 M. Huster and K. Smith, *Biochemistry*, 1990, **29**, 4348.
89 A.G.M. Chew, N.-U. Frigaard, and D.A. Bryant, *J. Bact.*, 2007, **189**, 6176.
90 J.B. Broderick, B.R. Duffus, K.S. Duschene, and E.M. Shepard, *Chem. Rev.*, 2014, **114**, 4229.

Natural Product Reports

HIGHLIGHT

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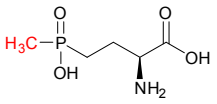
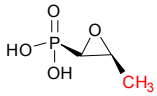


L-Phosphinothricin (PT)



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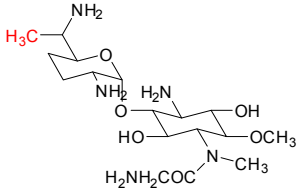
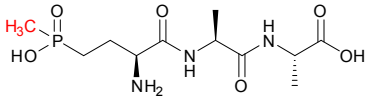
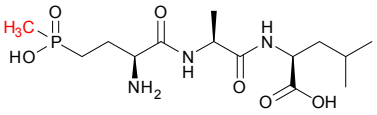


Fortimicin A



Bialaphos (L-PT-L-Ala-L-Ala)



Phosalacine (L-PT-L-Ala-L-Leu)

Natural Product Reports

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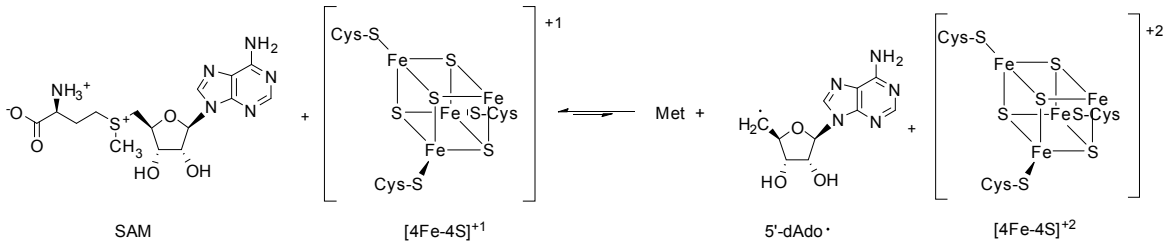

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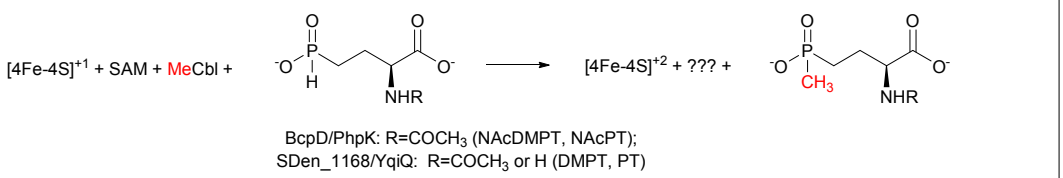

BcpD/PhpK: R=$COCH_3$ (NAcDMPT, NAcPT);
SDen_1168/YqiQ: R=$COCH_3$ or H (DMPT, PT)

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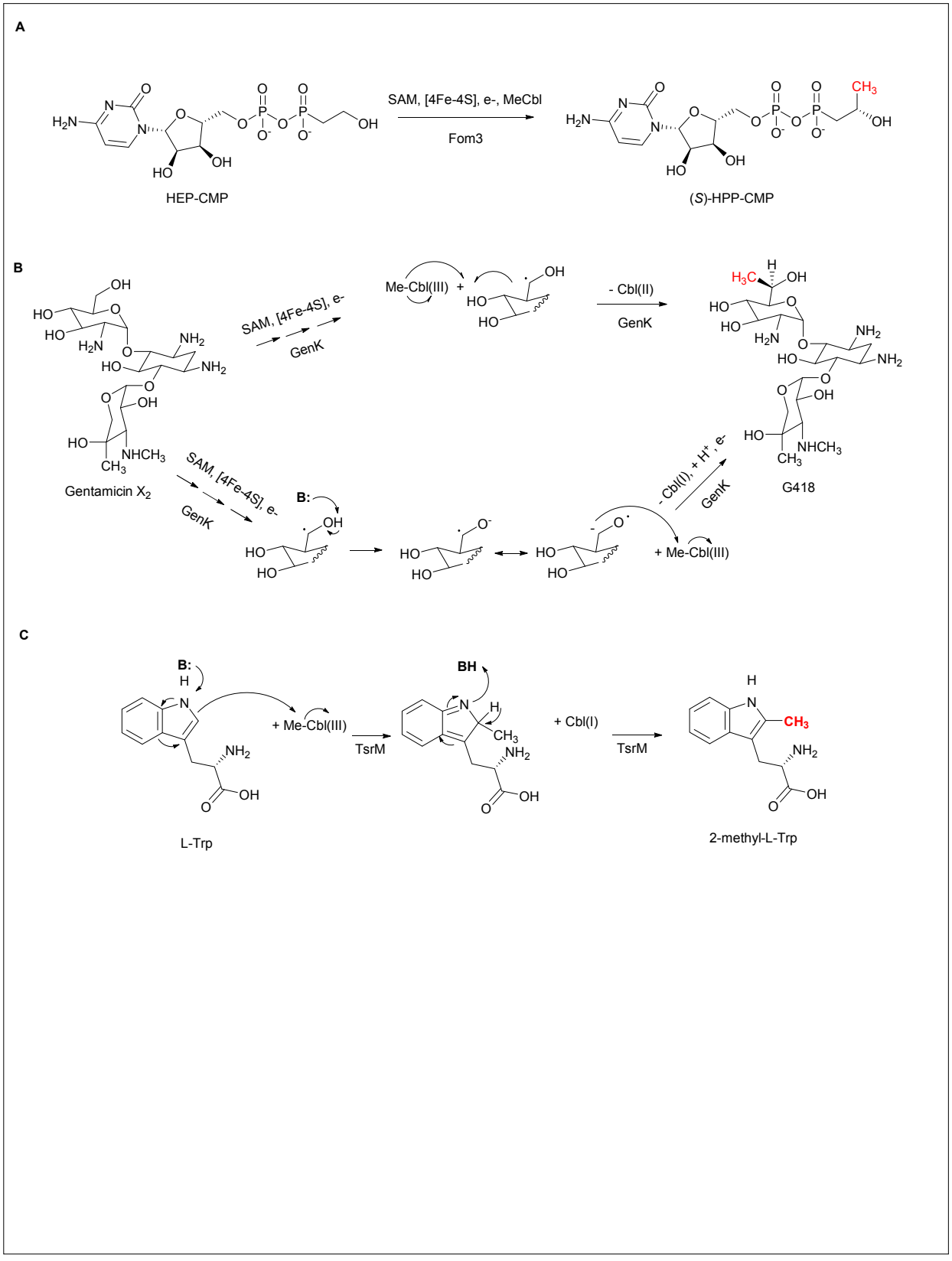

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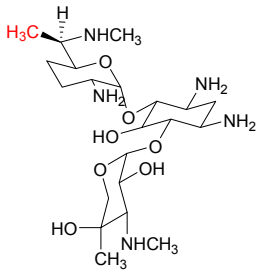
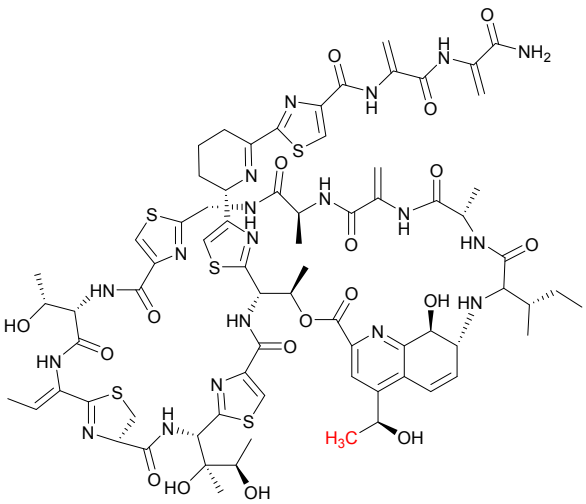


Gentamicin C_1



Thiostrepton A

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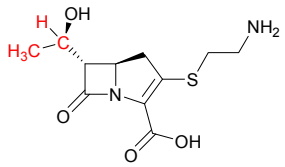
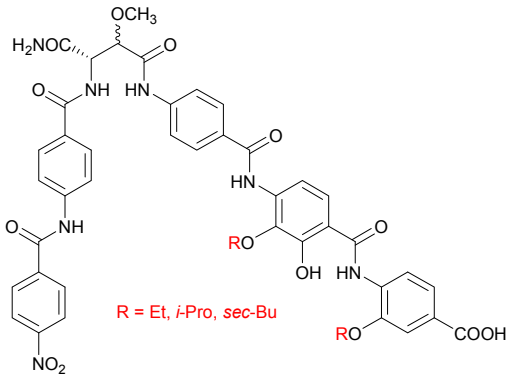
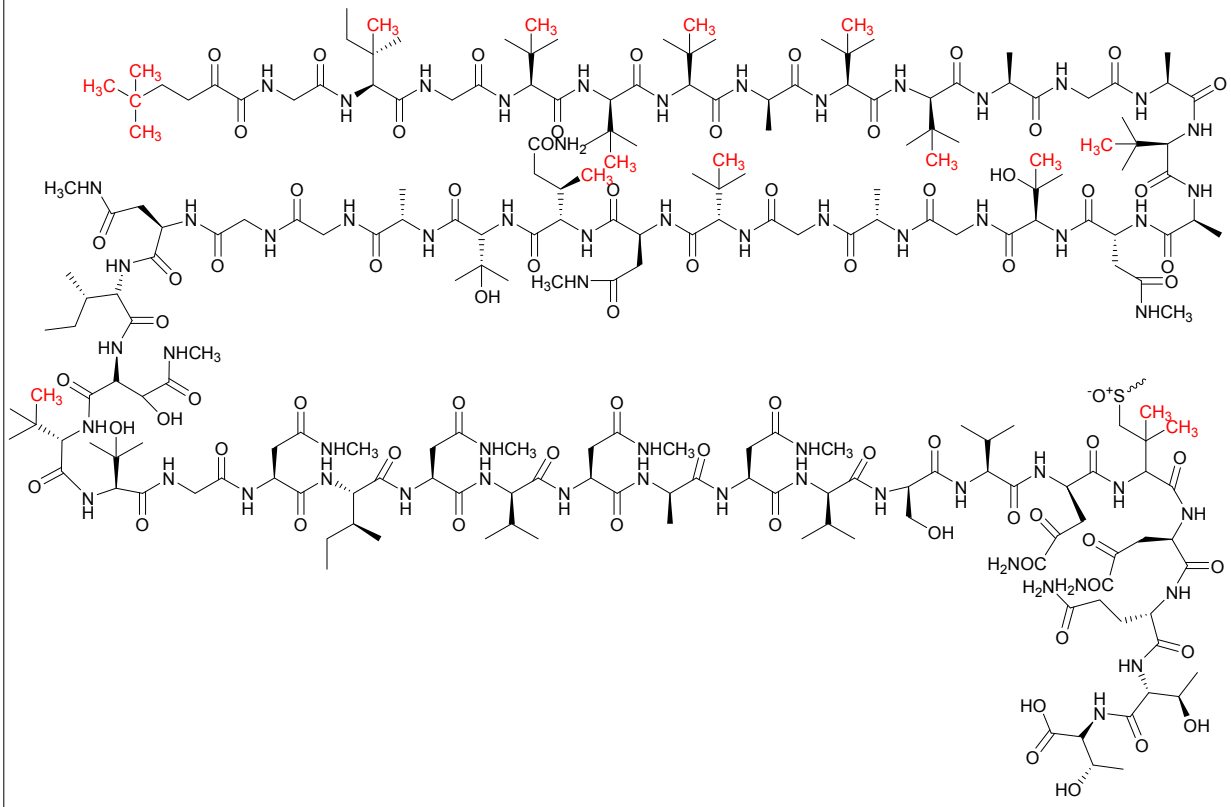


Thienamycin



Cystobactamids



Polytheonamides A and B (differing sulfoxide configurations)

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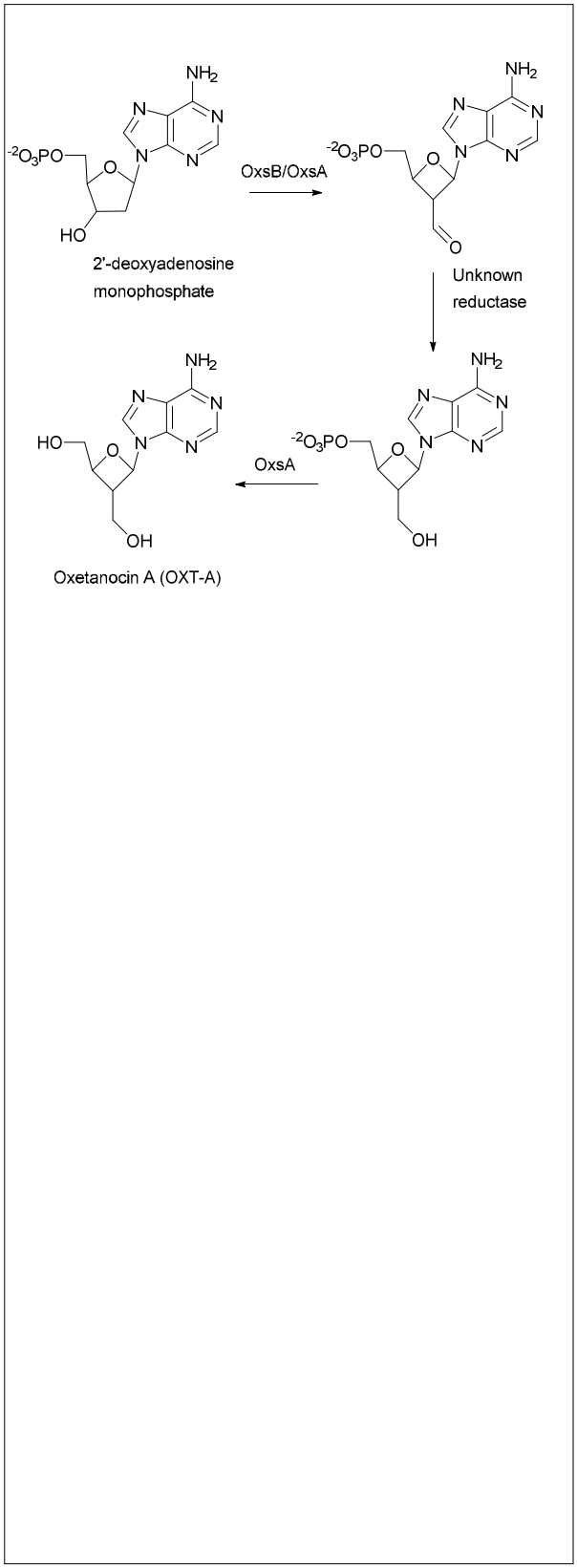

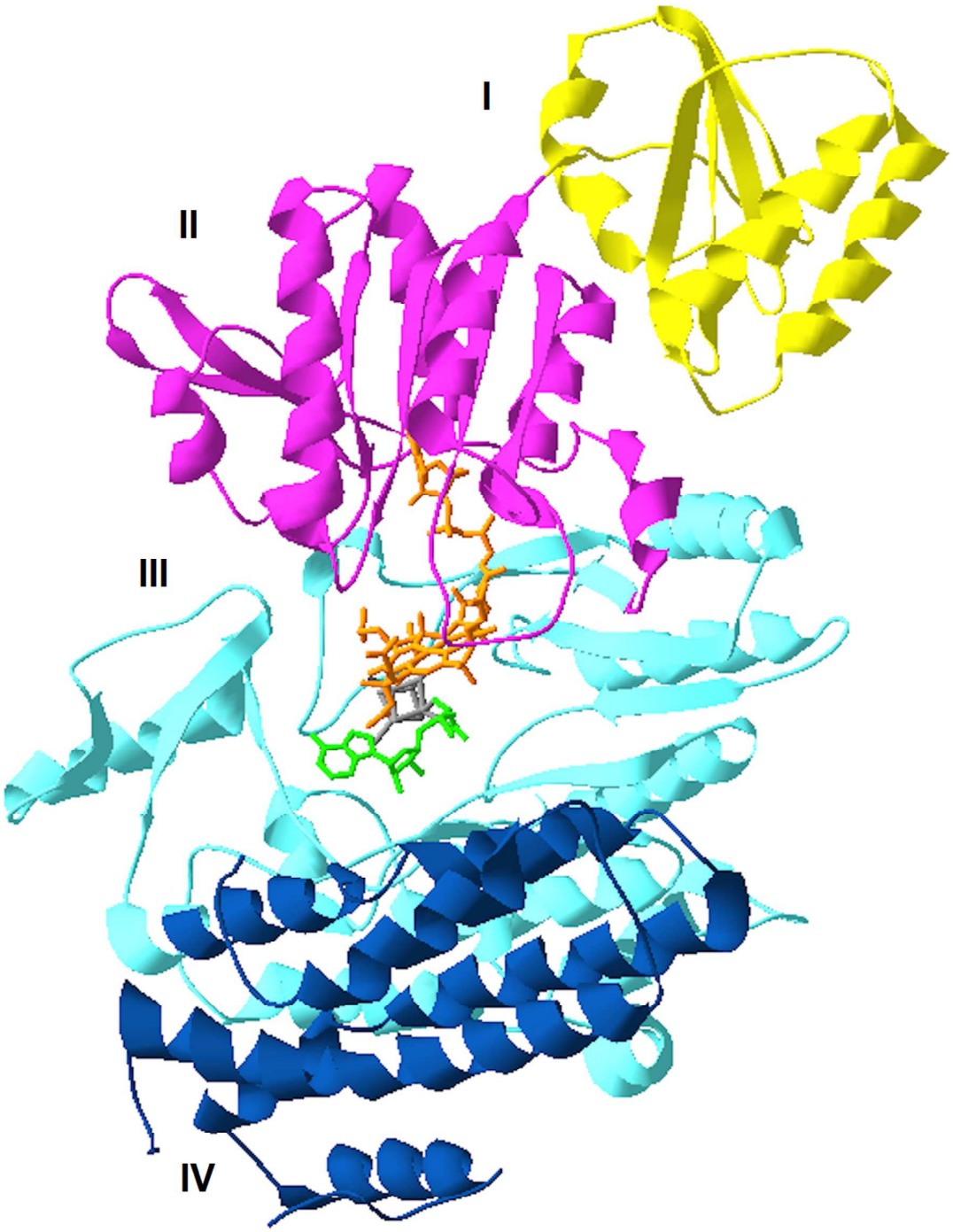
I
II
III
IV

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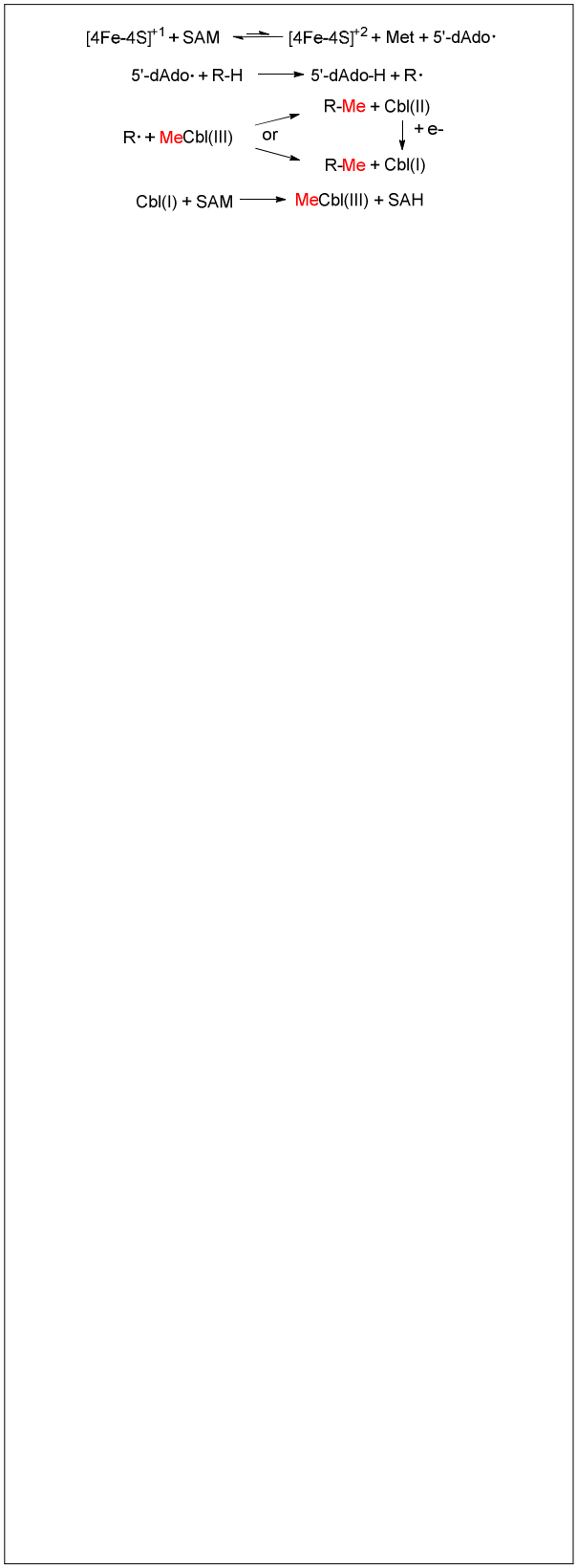

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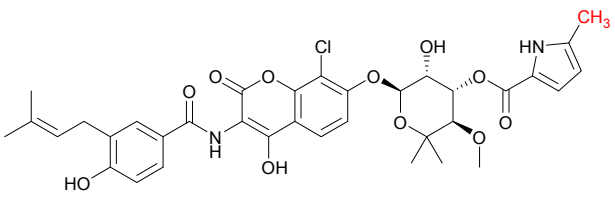
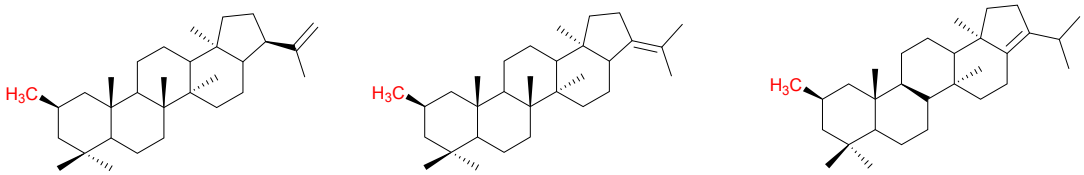
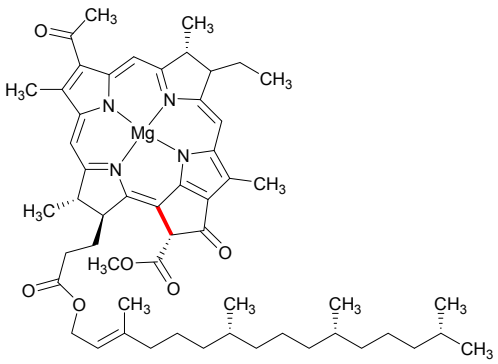


Clorobiocin



Hopanoids



Bacteriochlorophyll a